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## Species-specific modulation of the mitochondrial permeability transition by norbormide

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### Abstract

In the present study, we show that norbormide stimulates the opening of the permeability transition pore (PTP) in mitochondria from various organs of the rat but not of guinea pig and mouse. Norbormide does not affect the basic parameters that modulate the PTP activity since the proton electrochemical gradient, respiration, phosphorylation and  $\text{Ca}^{2+}$  influx processes are only partially affected. On the other hand, norbormide induces rat-specific changes in the fluidity of the lipid interior of mitochondrial membranes, as revealed by fluorescence anisotropy of various reporter molecules. Such changes increase the PTP open probability through the internal  $\text{Me}^{2+}$  regulatory site. The lack of PTP opening by norbormide is matched by a negligible perturbation of internal lipid domains in guinea pig and mouse, suggesting that the drug does not gain access to the matrix in the mitochondria from these species. Consistent with this interpretation, we demonstrate a preferential interaction of norbormide with the mitochondrial surface leading to alterations of the  $\text{Me}^{2+}$  binding affinity for the external PTP regulatory site. Our findings indicate that norbormide affects  $\text{Me}^{2+}$  binding to the regulatory sites of the PTP, and suggest that the drug could be taken up by a mitochondrial transport system unique to the rat. The characterization of the norbormide target may lead to a better understanding of the mechanisms underlying the mitochondrial PTP as well as to the identification of species-specific drugs that affect mitochondrial function.

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**Keywords:** Mitochondria; Norbormide; Permeability transition; Species-specificity

### 1. Introduction

Norbormide [5-( $\alpha$ -hydroxy- $\alpha$ -2-pyridylbenzyl)-7-( $\alpha$ -2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide] is a

vasoactive compound endowed with a unique species-specific constrictor activity that is restricted to the peripheral arteries of the rat [1–3]. In all tested arteries from other species, as well as in rat aorta and extravascular smooth muscle, norbormide exhibits vasorelaxant properties at concentrations that induce vasoconstriction in the rat peripheral arteries [3,4]. The mechanisms involved in these opposite effects of norbormide have not been clarified, but available evidence suggests that the vasoconstrictor effect may be mediated by the activation of a phospholipase C (PLC)-coupled receptor expressed in rat peripheral artery myocytes, whereas the relaxant effect may be the result of a reduction of  $\text{Ca}^{2+}$  entry through L-type  $\text{Ca}^{2+}$  channels [4–6].

**Abbreviations:** ANS, 8-anilino-1-naphthalene sulfonic acid; CsA, cyclosporine A; DPH, 1,6-diphenyl-1,3,5-hexatriene; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; Laurdan, 6-dodecanoyl-2-dimethyl-aminonaphthalene; MOPS, 4-morpholinepropanesulfonic acid; Norbormide, [5-( $\alpha$ -hydroxy- $\alpha$ -2-pyridylbenzyl)-7-( $\alpha$ -2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide]; RR, ruthenium red; PTP, permeability transition pore; *r*, fluorescence anisotropy

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A species-specific action of norbormide has also been observed at the level of mitochondrial function. Patil and Radhakrishnamurty [7] reported that low concentrations of norbormide alter the ATPase activity of mitochondria isolated from rat liver while no changes were observed in mice. The induction of matrix swelling and decrease in both succinate- and ATP-driven  $\text{Ca}^{2+}$  uptake in rat liver mitochondria were also reported, but the mechanistic basis for these effects of norbormide, and their relevance, remained unclear. In recent years, mitochondrial swelling and other functional alterations have been frequently associated to the opening of the permeability transition pore (PTP), a  $\text{Ca}^{2+}$ -activated, cyclosporin (CsA)-sensitive channel of the inner membrane. The sequence of events triggered by the opening of the PTP (dissipation of transmembrane electrochemical gradients, disruption of ionic homeostasis, mitochondrial swelling and massive ATP hydrolysis by the  $\text{F}_1\text{F}_0$ -ATPase) led to assume that the PTP may play an important role in the pathways to cell death [8–10].

In this paper, we have re-investigated the effects of norbormide on mitochondrial function, paying particular attention to the regulation of the PTP. Due to the species- and tissue-selectivity of norbormide as a toxicant, mitochondria from different rat tissues (liver, heart, kidney) and from different species (rats, mice and guinea pigs) were investigated. We found that the mitochondrial swelling induced by norbormide was due to the activation of the PTP. Surprisingly, this effect was specific for rat mitochondria. We show that norbormide affects  $\text{Me}^{2+}$  binding to the regulatory sites of the PTP through changes in the membrane fluidity, and suggest that the drug could gain access to the matrix by a mitochondrial transport system unique to the rat.

## 2. Materials and methods

Norbormide (Fig. 1) was a kind gift of I.N.D.I.A. Industria Chimica, Padova (Italy). 1,6-diphenyl-1,3,5-hexatriene (DPH), 8-anilinoanthracene-1-sulfonic acid (ANS), and carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone

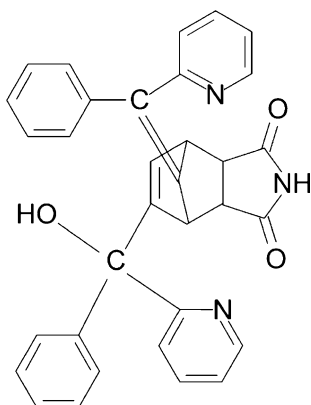


Fig. 1. Chemical structure of norbormide.

(FCCP) were products of Sigma. 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) was purchased from Molecular Probes. Other chemicals used were of analytical reagent grade.

Albino Wistar rats, CD1 mice and albino guinea pigs were used. All animals were purchased from Charles River, Italy.

Mitochondria from different tissues (liver, heart, and kidney) were prepared according to standard differential centrifugation procedures. The final pellet was suspended in Tris/HCl buffer, pH 7.4, containing 0.25 M sucrose and 0.1 mM EGTA, to give a protein concentration of 80–100 mg/ml, as measured using the Biuret method.

Mitochondrial oxygen consumption was measured polarographically in a thermostated ( $T=25^\circ\text{C}$ ), water-jacketed vessel, using a Clark electrode connected to a recorder.

Mitochondrial volume changes were measured as a decrease in the  $90^\circ$  light scattering of the mitochondrial suspensions at 540 nm, using a Perkin-Elmer LS-50B spectrophotofluorimeter equipped with magnetic stirring and thermostatic control.

Extramitochondrial  $\text{Ca}^{2+}$  was measured fluorimetrically using Calcium Green-5N, a membrane impermeant probe, which exhibits an increase in fluorescence emission intensity upon  $\text{Ca}^{2+}$  binding (excitation emission  $\lambda$ : 480–530 nm).

Fluidity changes of mitochondrial membranes promoted by norbormide were evaluated by the changes in fluorescence anisotropy of DPH, Laurdan and ANS, which bind hydrophobic regions and polar heads/lipid backbone interfaces, and surface areas of the membrane lipid bilayer, respectively [11–13]. ANS (final concentration 6  $\mu\text{M}$ ) was added to the stirred mitochondrial suspensions (0.2 mg/ml) and left to incubate for 2 min before measuring anisotropy. DPH- and Laurdan-labelled mitochondria were prepared by treating mitochondrial suspensions (5 mg/ml) with 200  $\mu\text{M}$  probe. After 20-min incubation at  $T=25^\circ\text{C}$ , the mitochondrial suspension was diluted to 0.2 mg/ml for anisotropy measurements. The fluorescence anisotropy ( $r$ ) was collected at 340 nm ( $\lambda_{\text{em}}=460$  nm) for DPH, at 350 nm ( $\lambda_{\text{em}}=435$  nm) for Laurdan, and at 360 nm ( $\lambda_{\text{em}}=480$  nm) for ANS, by calculating the  $I_{\text{VV}}$  and  $I_{\text{VH}}$ , i.e. the fluorescence intensities polarized parallel and perpendicular to the vertical plane of polarization of the excitation beam respectively. The anisotropy ( $r$ ) is defined by the equation  $r=(I_{\text{VV}}-GI_{\text{VH}})/(I_{\text{VV}}+2GI_{\text{VH}})$ , where  $G=I_{\text{HV}}/I_{\text{HH}}$  is the correction factor for instrumental artifacts.

For all measurements, mitochondria were incubated in a medium containing 10 mM Tris-MOPS, pH 7.4, 20  $\mu\text{M}$  EGTA-Tris, 1 mM  $\text{P}_i$ , 3  $\mu\text{g/ml}$  oligomycin, 2  $\mu\text{M}$  rotenone, 5 mM succinate and 200 mM sucrose (standard medium).

## 3. Results

We first investigated the effects of norbormide on mitochondrial respiration and energy coupling by measuring basal and ADP- and FCCP-stimulated oxygen consumption

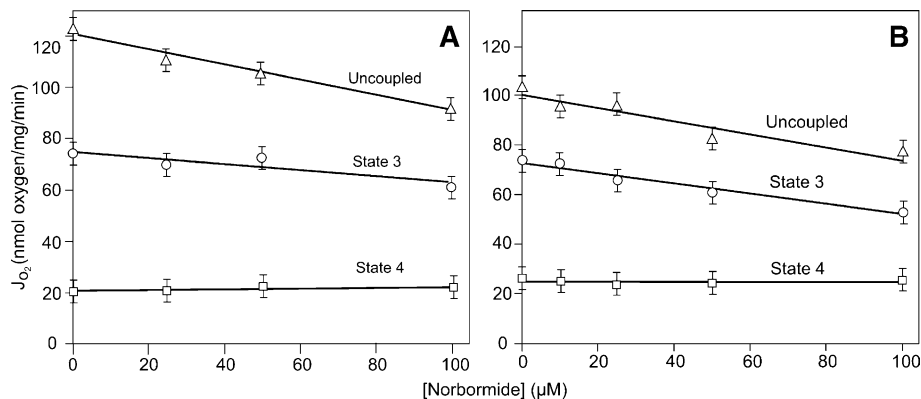


Fig. 2. Effect of norbormide on respiration of rat (A) and mouse (B) liver mitochondria. Mitochondria (0.5 mg/ml) were suspended at 25 °C in the standard medium containing succinate (5 mM) as the substrate plus 1  $\mu$ M CsA. After a 5-min incubation with norbormide, the  $O_2$  consumption was followed as described in Materials and methods. Mitochondrial respiration ( $J_{O_2}$ ) in state 4 ( $\square$ ), state 3 ( $\circ$ ) (0.3 mM ADP), or uncoupled ( $\triangle$ ) (0.1  $\mu$ M FCCP) is plotted versus the concentration of norbormide. Data are expressed as mean ( $\pm$ S.D.) of three independent determinations.

in the presence of succinate as the substrate. As shown in Fig. 2, norbormide did not affect the basal respiration of rat (A) and mouse (B) liver mitochondria at concentrations up to 100  $\mu$ M, whereas ADP- and FCCP-stimulated respiration was partially inhibited at drug concentrations higher than 30  $\mu$ M. Analogous experiments carried out with NADH-dependent substrates such as glutamate/malate revealed an inhibition (less than 30%) of norbormide on ADP-stimulated and uncoupled respiration only at drug concentrations  $>40$   $\mu$ M (data not shown). Similar results were obtained for mitochondria from guinea pig liver.

We next examined the influence of norbormide on the permeability transition of mitochondrial membranes. One milligram of liver mitochondrial protein was incubated at 25 °C in 2 ml of the standard medium. In the absence of  $Ca^{2+}$ , up to 100  $\mu$ M norbormide did not induce changes of mitochondrial volume in any of the species examined, as demonstrated by the constancy of the light scattering intensity at 540 nm over a 30-min incubation period (results not shown). In the experiments of Fig. 3, energized rat (panel A), mouse and guinea pig (panel B) liver mitochondria were loaded with 30  $\mu$ M, 80  $\mu$ M and 30  $\mu$ M  $Ca^{2+}$ , respectively, concentrations that per se were not sufficient to produce alterations in the mitochondrial membrane perme-

ability (traces a). However, upon the addition of 50  $\mu$ M norbormide, a large amplitude swelling of rat mitochondria occurred (panel A, trace b), which was completely prevented by 1  $\mu$ M cyclosporin A (CsA) (panel A, trace c), suggesting that norbormide induced the opening of the PTP [14]. Pore opening was also observed when the mitochondria were supplemented with norbormide before the addition of  $Ca^{2+}$ . The rate of the process was dependent on the drug concentration and the incubation time, and the maximal effect was reached at  $\geq 5$  min of incubation. Interestingly, the same norbormide concentrations were unable to open the PTP in mouse and guinea pig liver mitochondria (panel B, trace b) suggesting that its effects were species specific.

We next investigated the influence of norbormide on mitochondrial  $Ca^{2+}$  loading (Fig. 4). In the experiments illustrated in panel A, rat liver mitochondria were loaded with a train of 20  $\mu$ M  $Ca^{2+}$  pulses at 1-min intervals. Each  $Ca^{2+}$  addition gave rise to rapid increases of the Calcium Green-5N fluorescence, followed by a return to the original steady-state value as  $Ca^{2+}$  was taken up by mitochondria. When the loading threshold of about 120 nmol of  $Ca^{2+}$ /mg protein was reached, a net release of  $Ca^{2+}$  into the medium was observed (panel A, trace a). This fast process of  $Ca^{2+}$

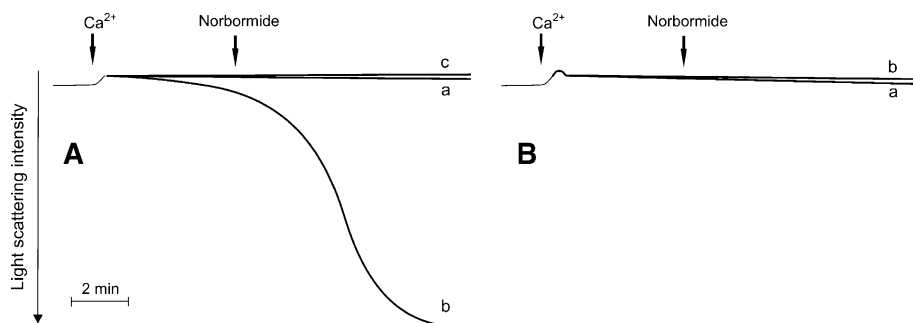


Fig. 3. Effect of norbormide on PTP opening in rat (A), mouse and guinea pig (B) liver mitochondria. Mitochondria (0.5 mg/ml) were incubated at 25 °C in the standard medium. PTP opening was monitored as the 90° light scattering decrease at 540 nm. Where indicated (arrows), the medium was supplemented with: traces a, 30  $\mu$ M (rat; panel A), 80  $\mu$ M (mouse; panel B), 30  $\mu$ M (guinea pig; panel B)  $Ca^{2+}$ ; traces b,  $Ca^{2+}$  plus 50  $\mu$ M norbormide. In trace c (panel A), 1  $\mu$ M CsA was added to the medium before the addition of  $Ca^{2+}$  and norbormide.

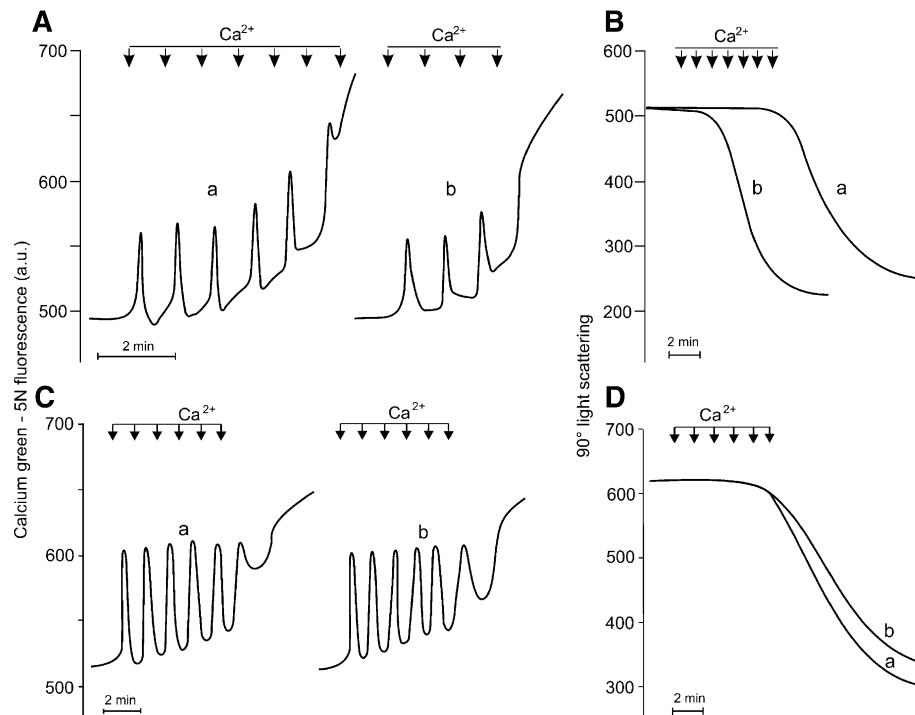


Fig. 4. Effect of norbormide on the  $\text{Ca}^{2+}$  retention capacity of rat and mouse liver mitochondria. The experiments were started by the addition of 1 mg/ml mitochondria (not shown) to the standard incubation medium. (A, B) Rat liver mitochondria in the absence (traces a) or presence (traces b) of 50  $\mu\text{M}$  norbormide were loaded with a train of 20  $\mu\text{M}$   $\text{Ca}^{2+}$  pulses at 1-min intervals. In the experiment of traces b, mitochondria were pre-incubated for 5 min with norbormide before the addition of  $\text{Ca}^{2+}$ . (A) Extramitochondrial  $\text{Ca}^{2+}$  was monitored as the fluorescence emission of 0.5  $\mu\text{M}$  Calcium Green-5N ( $\lambda_{\text{excitation}}=480$  nm;  $\lambda_{\text{emission}}=530$  nm). (B) Mitochondrial volume changes were measured from the  $90^\circ$  light scattering changes at 540 nm. (C, D) The experiments with mouse liver mitochondria were performed as described above except that  $\text{Ca}^{2+}$  pulses of 30  $\mu\text{M}$  were added at 1.5-min intervals.

release was due to the opening of the PTP because (i) it was accompanied by swelling (panel B, trace a); and (ii) the  $\text{Ca}^{2+}$  threshold was drastically increased in the presence of CsA (not shown). Incubation with 50  $\mu\text{M}$  norbormide for 5 min decreased the  $\text{Ca}^{2+}$  load required for PTP opening to 60 nmol of  $\text{Ca}^{2+}$ /mg protein without affecting the rate of  $\text{Ca}^{2+}$  uptake (panels A and B, traces b). In contrast to rat liver mitochondria, norbormide did not influence the  $\text{Ca}^{2+}$  retention capacity of mouse and guinea pig liver mitochondria. Indeed, the experiments illustrated in Fig. 4 show that  $\text{Ca}^{2+}$  release from mouse mitochondria (panel C) occurred after mitochondrial loading with six pulses of 30  $\mu\text{M}$   $\text{Ca}^{2+}$  at

1.5-min intervals, both in the absence (trace a) and the presence (trace b) of 50  $\mu\text{M}$  norbormide. In both cases, inhibition by 1  $\mu\text{M}$  CsA indicated that  $\text{Ca}^{2+}$  release was PTP dependent. Consistently, the onset of PTP-dependent mitochondrial swelling (panel D, trace a) was only negligibly affected by norbormide (trace b). Fig. 5 compares the concentration dependence of the effects of norbormide on the  $\text{Ca}^{2+}$  retention capacity of rat (panel A), mouse and guinea pig (panel B) liver mitochondria. Norbormide decreased the  $\text{Ca}^{2+}$  retention capacity of rat mitochondria in the concentration range 2–100  $\mu\text{M}$ , the maximal effect being reached at approximately 30  $\mu\text{M}$ . In striking contrast,

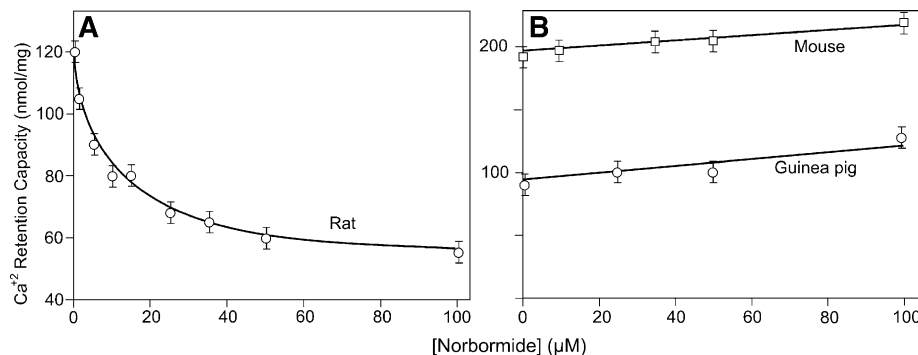


Fig. 5.  $\text{Ca}^{2+}$  retention capacity of rat and mouse and guinea pig liver mitochondria at increasing norbormide concentrations. The  $\text{Ca}^{2+}$  retention capacity of rat (A) and mouse and guinea pig (B) mitochondria was calculated according to the experimental procedure described in the legend to Fig. 4. Data are expressed as mean ( $\pm$ S.D.) of six independent determinations.

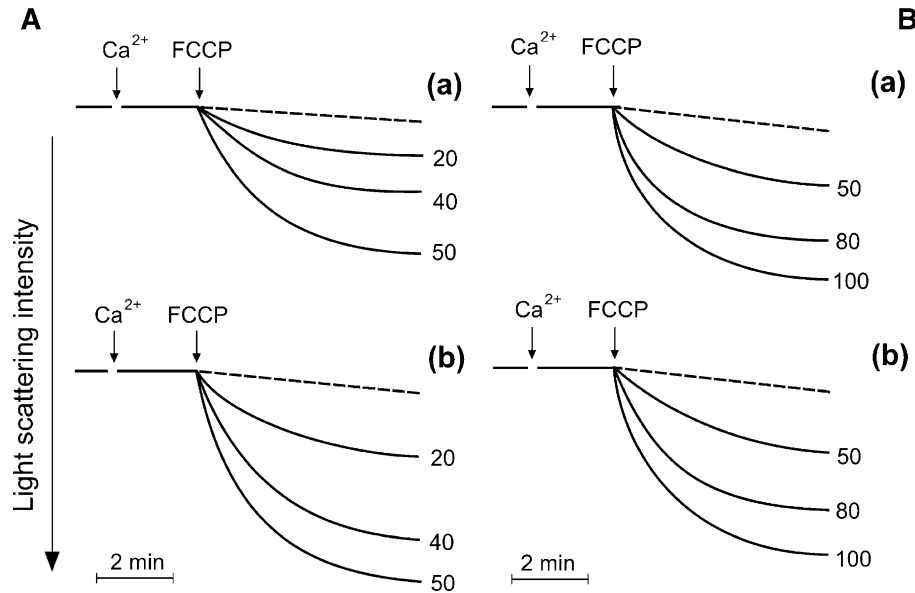


Fig. 6. Effect of the  $\text{Ca}^{2+}$  load on uncoupler-induced PTP in the absence and presence of norbormide. Liver mitochondria (1 mg/ml) from rat (A) or mouse (B) were incubated for 5 min in the standard medium (not shown) in the absence (traces a) or presence (traces b) of 35  $\mu\text{M}$  norbormide. Then, the indicated micromolar concentrations of  $\text{Ca}^{2+}$  were added, followed after further 2 min by 0.4  $\mu\text{M}$  FCCP. In the experiments depicted with broken lines, the same protocols were carried out in the presence of 1  $\mu\text{M}$  CsA.

the same concentration range of norbormide had no effect on the  $\text{Ca}^{2+}$  retention capacity of mouse and guinea pig mitochondria.

The results obtained with heart and kidney mitochondria were identical to those obtained with liver mitochondria of the three species, indicating that norbormide-stimulated PTP opening in rat, and the lack of activity of the drug in guinea pig and mouse, were not tissue-specific phenomena.

The next experiments were aimed at defining the influence of norbormide on the regulatory properties exerted by the two PTP regulatory metal ion ( $\text{Me}^{2+}$ ) binding sites in mitochondria. Current evidence suggests that two  $\text{Me}^{2+}$  regulatory sites can be defined, (i) an internal site: the occupancy of this site by  $\text{Ca}^{2+}$  increases the PTP “open” probability, while other  $\text{Me}^{2+}$  ( $\text{Sr}^{2+}$ ,  $\text{Mn}^{2+}$ ) have an inhibitory effect, and (ii) an external site: when this site is

occupied by any  $\text{Me}^{2+}$ , including  $\text{Ca}^{2+}$ , the PTP opening probability decreases. By following the experimental protocol described by Bernardi et al. [15], the features of the two  $\text{Me}^{2+}$  binding sites were studied in rat and mouse liver mitochondria, both in the absence and the presence of norbormide.

For studies involving the internal  $\text{Me}^{2+}$  ( $\text{Ca}^{2+}$ ) site, mitochondria (1 mg/ml) were suspended in the standard medium both in the absence (control) and the presence of norbormide at a concentration of 35  $\mu\text{M}$  (the concentration which induced maximal effect on the PTP, Fig. 5). After 5 min of incubation, the mitochondria were supplemented with  $\text{Ca}^{2+}$  pulses of increasing size, but in a range of concentrations that ensured a full retention of the ion (less than 60 nmol/mg for rat and 180 nmol/mg for mouse mitochondria; see Fig. 5). Two minutes after  $\text{Ca}^{2+}$  uptake,

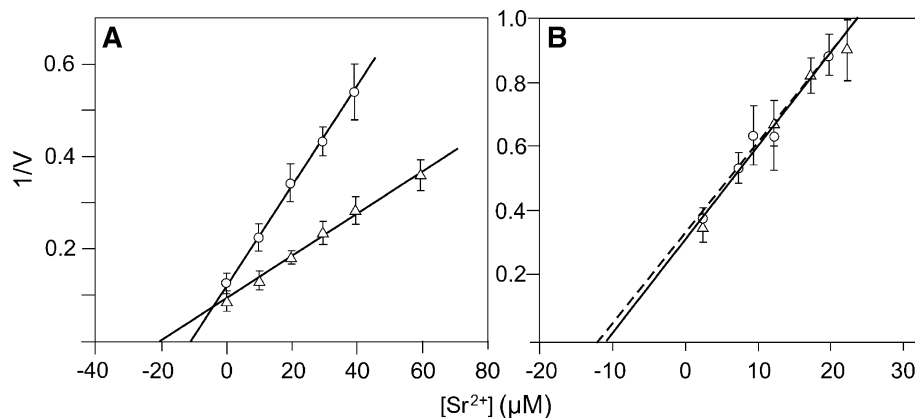


Fig. 7. Dixon plot for  $\text{Sr}^{2+}$  inhibition of uncoupler-induced PTP in the absence (O–O) and presence (Δ–Δ) of norbormide. Experimental conditions were as in Fig. 6, except that mitochondria were loaded with a fixed amount of  $\text{Ca}^{2+}$ : 30  $\mu\text{M}$  in rat (A) and 80  $\mu\text{M}$  in mouse (B) and after 2 min supplemented with variable sizes of  $\text{Sr}^{2+}$  (in the range 10–60  $\mu\text{M}$ ) before the addition of FCCP. Data are expressed as mean ( $\pm$ S.D.) of three independent determinations.

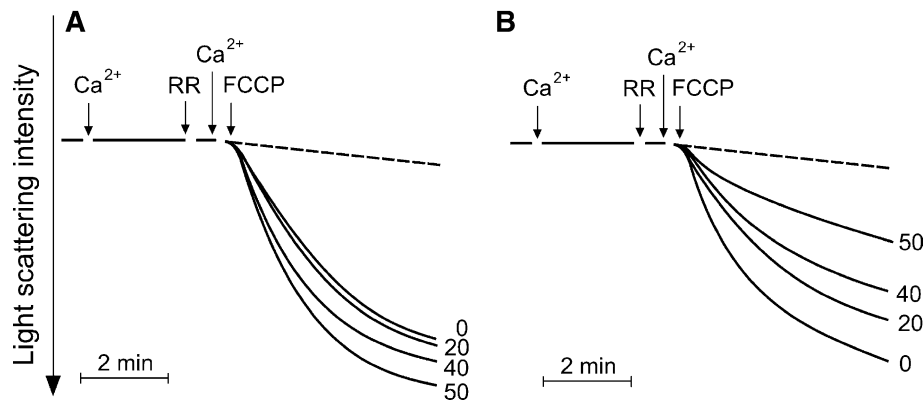


Fig. 8. Effect of norbormide on uncoupler-induced PTP in the presence of external  $\text{Ca}^{2+}$ . Mitochondria (1 mg/ml) from rat (A) or mouse (B) were incubated in the standard medium (not shown) in the absence or presence of the indicated micromolar concentrations of norbormide. After 5 min, 30  $\mu\text{M}$  (panel A) or 80  $\mu\text{M}$   $\text{Ca}^{2+}$  (panel B) was added (first arrow), followed by 0.1  $\mu\text{M}$  RR, 250  $\mu\text{M}$   $\text{Ca}^{2+}$ , and 0.4  $\mu\text{M}$  FCCP. In the experiments depicted with broken lines, the same protocols were carried out in the presence of 1  $\mu\text{M}$  CsA.

FCCP (0.4  $\mu\text{M}$ ) was added and the PT was measured as the decrease of  $90^\circ$  light scattering. In agreement with previous data [15], the rate of swelling induced by FCCP in both rat (Fig. 6, panel A, trace a) and mouse (Fig. 6, panel B, trace a) mitochondria was essentially determined by the amount of accumulated  $\text{Ca}^{2+}$ , with an increase that matched the size of the  $\text{Ca}^{2+}$  load. In the presence of norbormide, the uncoupler-induced permeabilization process was much faster in rat mitochondria (panel A, trace b) whereas no effect was observed in mouse (panel B, trace b) mitochondria. The stimulating effect of norbormide on the internal  $\text{Me}^{2+}$  binding site could also be observed after loading rat mitochondria with a PTP inhibitory  $\text{Me}^{2+}$  such as  $\text{Sr}^{2+}$ . The experimental conditions were the same as previously described, except that mitochondria, after taking up a small load of  $\text{Ca}^{2+}$ , were supplemented with increasing  $\text{Sr}^{2+}$  loads before the addition of FCCP. The results were analysed according to a Dixon plot. As shown in Fig. 7A, the value of the  $\text{Sr}^{2+}$  inhibition constant ( $K_i$ ) was increased approximately two-fold (from  $\sim 10$  to  $\sim 20$   $\mu\text{M}$ ) in the presence of norbormide. In contrast, a

negligible effect on the  $K_i$  was observed in mouse mitochondria (panel B).

The results shown in Fig. 8 illustrate how norbormide affects the PTP opening induced by FCCP in the presence of external metal ( $\text{Ca}^{2+}$  in this case), both in rat (panel A) and mouse (panel B) mitochondria. After the accumulation of a  $\text{Ca}^{2+}$  pulse that did not cause pore opening per se, ruthenium red (RR) was added to prevent further uptake of externally added  $\text{Ca}^{2+}$ , followed by a further  $\text{Ca}^{2+}$  addition and, finally, FCCP. The experiments were performed in the absence or presence of the indicated concentrations of norbormide. A comparison between norbormide-free and norbormide-treated rat mitochondria shows that the FCCP-triggered PTP opening was slightly accelerated as the drug concentration was increased (panel A). In striking contrast, norbormide strongly reduced the rate and amplitude of FCCP-induced PTP-dependent swelling in mouse mitochondria (panel B). To better understand this effect, we investigated whether the PTP-regulatory properties of the external  $\text{Me}^{2+}$  site were affected by norbormide. The experiments were performed at fixed norbormide concentrations and different loads of

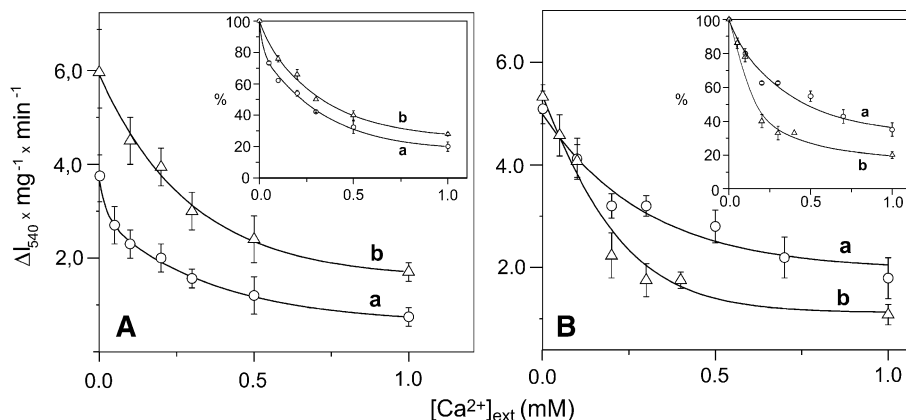


Fig. 9. Inhibition of uncoupler-induced PTP by external  $\text{Ca}^{2+}$  in the absence and presence of norbormide. Experimental conditions were as described in the legend to Fig. 7, except that norbormide concentration was kept constant and the external  $\text{Ca}^{2+}$  was varied as indicated in the abscissa. Panel A: rat liver mitochondria; Panel B: mouse liver mitochondria. In traces a, norbormide was not present; in traces b, mitochondria were supplemented with 35  $\mu\text{M}$  norbormide. Data are shown as mean ( $\pm$ S.D.) of four independent determinations.



Table 1

Fluorescence anisotropy ( $r_o$ ) of DPH-, Laurdan- and ANS-labelled rat and mouse mitochondria

$r_o$	Rat mitochondria	Mouse mitochondria
DPH	$0.13 \pm 0.03$	$0.15 \pm 0.02$
Laurdan	$0.17 \pm 0.05$	$0.22 \pm 0.05$
ANS	$0.30 \pm 0.05$	$0.32 \pm 0.06$

external  $\text{Ca}^{2+}$  in a protocol identical to that described above. The resulting decreases in the rate of light scattering were measured and plotted as a function of  $[\text{Ca}^{2+}]_{\text{ext}}$ . Fig. 9 shows the patterns of PTP inhibition by externally added  $\text{Ca}^{2+}$  for rat (A) and mouse (B) mitochondria. After treatment with 35  $\mu\text{M}$  norbormide, the external  $\text{Ca}^{2+}$  concentration required for 50% inhibition ( $I_{50}$ ) in rat mitochondria (panel A, inset) shifted from a value of  $\sim 0.2$  mM (trace a) (see also Ref. [15]) to  $\sim 0.3$  mM (trace b). Thus, norbormide exhibited a moderate antagonizing effect on the PTP-inhibitory ability exerted by  $\text{Ca}^{2+}$  at the external  $\text{Me}^{2+}$  binding site. In contrast, the  $I_{50}$  of mouse mitochondria (panel B, inset), decreased from  $\sim 0.5$  mM (trace a) to  $\sim 0.2$  mM (trace b), indicating that norbormide strongly enhanced the inhibitory action exerted by  $\text{Ca}^{2+}$  at the same site. The effects of norbormide were also observed when  $\text{Mg}^{2+}$  rather than  $\text{Ca}^{2+}$  was used in identical protocols, with a shift of the  $I_{50}$  from  $\sim 0.65$  mM to  $\sim 0.12$  mM in mouse mitochondria. It must also be mentioned that the effects of norbormide on mouse mitochondria did not change if the drug was added immediately prior to the external  $\text{Me}^{2+}$  rather than at the beginning of the incubations. Interestingly, norbormide-induced perturbation of the external  $\text{Me}^{2+}$  binding site was not seen if the drug was added after the  $\text{Me}^{2+}$  (results not shown).

In order to test whether the effects of norbormide were related to changes of the dynamic properties of mitochondrial membranes, we analyzed the changes in the steady-state fluorescence anisotropy of mitochondria-bound DPH, Laurdan and ANS, which report on the fluidity properties of different domains of the lipid bilayer [11–13,16]. The initial anisotropy values ( $r_o$ ) of the three probes in rat and mouse mitochondria are shown in Table 1. As seen in Fig. 10, strong perturbations of both the internal lipid domains (as

sensed by DPH) and polar head group/hydrophobic tail border areas (as sensed by Laurdan) were observed in rat mitochondria in the presence of increasing concentrations of norbormide. The final effect was an increase in membrane fluidity, as deduced by the decrease of the fluorescence anisotropy of the probes. In contrast, only slight changes in the fluorescence anisotropy of DPH- and Laurdan-labelled mouse mitochondria were noticed, suggesting that, in the mouse, norbormide interacted only marginally with mitochondrial domains buried in the internal core of the membrane or localized in the hydrophobic tail/polar head lipid interfaces. The mitochondrial regions facing the bulk medium, sensed by ANS anisotropy, were differentially affected by norbormide in the different species. Indeed, a decrease in lipid fluidity was observed for rat mitochondria, whereas norbormide rendered the surface membrane areas more fluid in mouse mitochondria (Fig. 10).

Under all experimental conditions, norbormide did not modify the fluorescence intensities of ANS, Laurdan and DPH, thus ruling out any effect on the membrane-binding properties of the probes (data not shown). The constancy of the fluorescence intensities also indicated that the probe lifetimes remained unchanged in the presence of norbormide, thus allowing to relate the anisotropy changes only to altered membrane fluidity. The fluorescence intensities of the probes were very similar in rat and mouse, which suggested that the amount of probe bound to mitochondria in the two species was the same.

#### 4. Discussion

In this paper, we have shown that norbormide affects the properties of the PTP in mitochondria from various tissues of the rat, but not guinea pig or mouse. Norbormide did not affect the basic parameters that modulate the pore. In particular, (i) the respiratory capacity and coupling efficiency of norbormide-treated mitochondria were adequate to sustain the proton electrochemical gradient, as indicated by the maintenance of respiratory control; thus, a decreased proton electrochemical gradient cannot account for the present observations of PTP opening, and (ii) norbormide

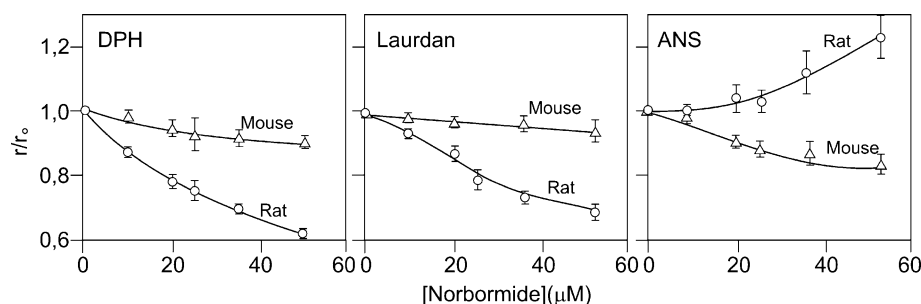


Fig. 10. Effect of increasing norbormide concentrations on the fluorescence anisotropy of DPH-, Laurdan- and ANS-labelled mitochondria. Mitochondria labelling was performed as indicated in Materials and methods. The fluorescence anisotropies ( $r$ ) were collected at 340 nm ( $\lambda_{\text{em}}=460$  nm) for DPH, at 350 nm ( $\lambda_{\text{em}}=435$  nm) for Laurdan, and at 360 nm ( $\lambda_{\text{em}}=480$  nm) for ANS. All anisotropy intensities were normalized to those observed before the addition of norbormide ( $r_o$ , see Table 1). Data are shown as mean ( $\pm$ S.D.) of three independent determinations.

did not affect  $\text{Ca}^{2+}$  uptake before the onset of the permeability transition, ruling out that the PTP opening is due to an alteration of  $\text{Ca}^{2+}$  transport.

Our results suggest that the modulation of the PTP by norbormide is exerted through changes in mitochondrial membrane fluidity, which appears to affect the accessibility of PTP  $\text{Me}^{2+}$  regulatory sites. The correlation between the stimulatory effect of the drug on the PTP and membrane dynamics has been inferred from studies with various reporter molecules, namely DPH, Laurdan and ANS. DPH is typically used to sense fluidity changes of the membrane hydrophobic lipid core, since this probe lies preferentially close to the  $\text{C}_{10}$ – $\text{C}_{12}$  region of the acyl chains [12]. Laurdan shows strong sensitivity to the water content and dynamics in lipid interfaces since its fluorescent moiety is located at the hydrophilic/hydrophobic interfaces of the phospholipid bilayer near the upper part of the lipid backbone region ( $\text{C}_1$ – $\text{C}_4$ ) [16]. ANS binds to membranes in the region of the phospholipid polar heads. The non-polar part of the ANS molecule is buried into the fatty acid alkyl chains, causing the center of the aromatic ring to lie approximately at the level of the phospholipid carbonyl group with the sulfonate group projecting towards the polar choline heads [11]. The data shown in Fig. 10 demonstrate that rat mitochondrial membranes are strongly affected by norbormide, which increases the fluidity of the internal domains and the headgroups/hydrophobic core border areas of the lipid bilayer. Such a fluidizing action may explain the amplification of the  $\text{Ca}^{2+}$  stimulatory effect on the PTP and the consequent reduction in the inhibitory efficiency of  $\text{Sr}^{2+}$ , possibly through secondary changes of the  $\text{Me}^{2+}$  binding affinity for the internal regulatory site [15]. Consistent with this suggestion, physical perturbation of mitochondrial membrane structure as a mechanism of PTP regulation has been described in other systems [17–20].

A correlation between changes in mitochondrial membrane fluidity and stimulation of PTP opening is supported by the results obtained with the other two tested animal species. Indeed, the internal mitochondrial membrane regions of mouse and guinea pig are insensitive to the perturbing effects of norbormide, probably because in these species, the drug is not able to cross the membrane barrier. According to this hypothesis, norbormide should rather distribute via polar interactions to the membrane surface, which is supported by the remarkable perturbation of surface membrane domains that leads to an increased  $\text{Me}^{2+}$  affinity for the external binding site. Actually, a relevant decrease in the values of  $I_{50}$  for both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  was observed, which reflects a strong enhancement of the PTP inhibitory potency of the  $\text{Me}^{2+}$ . These findings would suggest the existence of a rat-specific mitochondrial surface transport system that allows the internalization of the drug across the membranes. The result would be an increase of membrane lipid fluidity accompanied by a higher sensitization of the PTP to  $\text{Ca}^{2+}$ . The absence of

demonstrable effects of norbormide on the PTP of mouse and guinea pig suggests that the putative carrier is absent in the latter species, or shielded from contact with the drug due to different membrane structural arrangements. Alternative (or complementary) mechanisms for the norbormide effect are possible. For example, the opposite effects of the drug on the PTP- inhibitory ability at the external  $\text{Me}^{2+}$  site could be due to the binding of norbormide to the membrane surface or to the  $\text{Me}^{2+}$  site itself, which would affect the internal, PTP-stimulatory  $\text{Me}^{2+}$  site in different ways in the various animal species.

An intriguing question posed by the present results is whether the species-specific PTP modulation by norbormide and the species-specific toxicity are casually linked, as suggested by the fact that both processes are specific for the rat. Norbormide is a mixture of eight racemate diastereoisomers [21], which differ in their vasoconstrictory activity [21,22]. We therefore plan to extend our investigations to isomeric as well as to analog derivatives of norbormide in order to identify the most active elements on the PTP.

To the best of our knowledge, norbormide is the first example of a drug that affects the PTP in a species-specific manner. On the one hand, this result should induce some caution in extrapolating pharmacological data obtained in the rat to humans. On the other, it should stimulate investigations of other species-specific drugs that affect mitochondrial function.

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